The Occurrence of Enzymes Synthesising Flavin Mononucleotide(FMN) and Flavin-Adenine Dinucleotide (FAD) in Milk

Riboflavin functions metabolically as its coenzyme nucleotides, viz., flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD)[1]. The only reports in literature on the enzymic synthesis of these nucleotides pertain to the study of these enzymes in yeasts[2-4] and the enzyme synthesising FMN in plants[5]. In this communication the results of preliminary investigations on the occurence of these two enzymes in milk are reported.

Human milk was cooled immediately after collection. The cooled milk was centrifuged at 5000 r.p.m. for 30 min. at 0 to 5° C. The fat was removed and the skim milk after dialysis against distilled water for 18 hrs. with repeated changes of water was used as the enzyme preparation.

Cow's milk was cooled immediately after being collected directly into a sample flask. The cooled milk was centrifuged in cold (0 to 5°) for 30 min. at 5000 r.p.m. The fat free centrifugate was dialysed for 18 hrs. against distilled water. The dialysate was brought to 0·4 saturation of ammonium sulphate by addition of saturated solution of $(NH_4)_2SO_4$, in cold (0 to 2°). The precipitate obtained by centrifuging at 4000 r.p.m. for 30 min. in cold was dissolved in minimum amount of water and dialysed against distilled water till the dialysate gave a negative NESSLER's test for $(NH_4)^+$ ions. It was then centrifuged at 4000 r.p.m. and the supernatant was used as the enzyme preparation.

The enzymic synthesis of FMN from riboflavin was established as follows: A 10 ml. reaction mixture consisting of 0·5 ml. ATP (1×10^{-3} M), 0·5 ml. NaF (1×10^{-1} M), 0·5 ml. $MgSO_4$ (3×10^{-4} M), 1·0 ml. of riboflavin (1×10^{-4} M), 3·5 ml. of veronal HCl buffer, p_H 8·6 (0·1 M) and 4·0 ml. of enzyme preparation was incubated at 37° C. for 4 hrs. The reaction was stopped by adding 4 ml. of trichloroacetic acid (17·5% w/v) and heating at 80 to 85° C. for 10 min. The supernatant obtained by centrifuging at 2000 r.p.m. for 10 min. was subjected to preparative circular paper chromatography[6], using butanol:acetic acid:water (4:1:5) as the solvent. The eluate in water of the band corresponding to FMN showed absorption maxima characteristic of FMN and was chromatographically identical with an authentic sample of FMN. On enzymic as well as acid hydrolysis, riboflavin and phosphate were the only products formed of such degradation. Suitable aliquots of the reaction filtrates were analysed for FMN formed, by the circular chromatographic technique[4]. FMN was located on the chromatograms in u.v. light (R_f 0·29 to 0·30), the paper strips were eluated with glass distilled water and the fluorescence measured in a Klett fluorimeter. Appropriate controls were carried out. The flavokinase activity was observed in cow's milk but not in human milk. The enzyme functions optimally at a p_H of 8·6 and a temperature of 37° C.

The enzymic synthesis of FAD from FMN was established as follows: A 10 ml. reaction mixture containing ATP 0·5 ml. (1×10^{-3} M), 0·5 ml. NaF (1×10^{-1} M), 0·5 ml. $MgSO_4$ (3×10^{-4} M), 1·0 ml. FMN (1×10^{-4} M), 3·5 ml. buffer at p_H 7·6 veronal HCl (0·1 M) and 4·0 ml. of enzyme preparation, was incubated at 37° C for 2 hrs. The reaction was stopped by heating on a boiling water bath for 10 min. The aliquots from the supernatant obtained by centrifuging at 2000 r.p.m. for 10 min. were subjected to chromatography as for FMN. The eluate corresponding to FAD showed an absorption maxima characteristic of the substance. It was chromatographically identical with an authentic sample of FAD. On enzymic hydrolysis in the absence of NaF it gave FMN. After acid hydrolysis with 6N H_2SO_4 at 15 lbs. for 6 hrs. the acid free neutral hydrolysate after chromatography showed a pink band when sprayed with Bratton-Marshall reagent[7] indicating an adenine moiety. FAD was estimated by the method of GIRI and KRISHNASWAMY[6]. The enzymes from cow and human milk showed p_H optimum at 7·6 and temperature optimum at 37° C. Further work on these enzymes in milk is in progress.

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